Correction to: The Effect of Bacterial Sulfate Reduction Inhibition on the Production and Stable Isotopic Composition of Methane in Hypersaline Environments

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Abstract The aim of this research was to investigate the competition between methanogens and sulfate-reducing bacteria in hypersaline environments. Samples of photosynthetic microbial mats, both soft mats (salinities of 55–126 ppt) and gypsum-hosted endo-evaporite mats (salinities of 77–320 ppt), were obtained from hypersaline environments in

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California, USA, Mexico and Chile. Methane production was determined from the increase in headspace methane concentration within incubation vials containing mat samples. At the end of the incubation period, the $\delta^{13}\text{C}$ values of produced methane were measured. Soft microbial mat vials containing molybdate, a specific inhibitor of bacterial sulfate reduction, exhibited dramatically higher methane production rates and higher (enriched in ^{13}C) methane $\delta^{13}\text{C}$ values than the controls. This suggests that the inhibition of sulfate reduction allowed the methanogens at these sites to use the competitive substrates (H_2 and/or acetate) made available. Further, the higher $\delta^{13}\text{C}$ values of the produced methane suggest that substrates (both competitive and non-competitive) were used to near completion. At the endoevaporite sites, which have much higher salinities than the soft mat sites, methane production was not significantly different and the methane $\delta^{13}\text{C}$ values either remained the same or decreased (depleted in ^{13}C) with added molybdate. We suggest that substrate availability increased enough to allow for somewhat greater isotopic fractionation resulting in the lower methane $\delta^{13}\text{C}$ values that were observed, but not enough to significantly increase measured production rates. Where no changes in either methane production rates or $\delta^{13}\text{C}$ values occurred, we hypothesize that salinity itself was inhibiting sulfate reduction and thus controlling microbe populations and rates of metabolism.

Keywords Hypersaline · Methane production · Stable isotopes · Molybdate

Introduction

The microbial mats found in hypersaline environments have been studied for many years; they are modern analogues of some of Earth's earliest ecosystems (Des Marais 2003). Both soft microbial mats and gypsum-hosted endoevaporite microbial mats can occur within hypersaline environments. The soft mats dominate at lower salinity brines, while the endoevaporites occur at higher salinities. These microbial mats contain a consortium of microbial communities ranging from primary producers, to fermenters to sulfate reducers and methanogens, which perform essential cycling of elements (Des Marais 1995). Hypersaline environments have taken on added relevance to the astrobiology community, especially in light of the seasonal variability of methane found in the atmosphere of Mars (Webster et al. 2018), on whose surface salts and possible flowing brines have also been found (Osterloo et al. 2008; McEwen et al. 2011; Ojha et al. 2015).

The presence of methane and its stable isotope value have been suggested as biosignatures in the search for life on other planets (Yung et al. 2018). Biologically produced methane can be identified by its stable isotopic composition as it is typically more depleted in ^{13}C relative to methane from other sources. Individual measurements of the isotopic composition of biogenic methane range from -110 to -50‰ (Whiticar 1999), where values are measured in the familiar delta notation:

$$\delta^{13}\text{C} \text{ (in ‰)} = ((R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}}) \times 1000$$

where R is the ratio of $^{13}\text{C}/^{12}\text{C}$. Thermogenic methane, produced from alteration of organic matter under high temperature and pressures (i.e., natural gas), tends to have $\delta^{13}\text{C}$ values more enriched in ^{13}C , generally ranging from -50 to -20‰ (Whiticar 1999).

Both sulfate reduction and methane production are important terminal electron-accepting processes during anaerobic respiration. When sulfate is available, as in marine environments, sulfate-reducing bacteria will out-compete the methanogens for the competitive

substrates, H₂ and acetate (e.g., Oremland and Polcin 1982). As a result, methane that is produced under high-sulfate conditions tends to come from the non-competitive substrates (i.e., methylamines, dimethylsulfide and methanol) (e.g., King et al. 1983).

The work described here investigated the relationship between bacterial sulfate reducers and methanogens in hypersaline environments by inhibiting sulfate reduction with sodium molybdate, a known inhibitor of bacterial sulfate reduction (Oremland and Capone 1988). In hypersaline mat environments, it has been shown that methanogens use the methylamines as their main substrates (e.g., Kelley et al. 2015); however, it is unclear that they use the non-competitive substrates because of competition with the bacterial sulfate reducers, or because the methanogens capable of using the competitive substrates are not present at these salinities (e.g., Oren 2011). We therefore hypothesized that under these experimental conditions, if an increase in methane production occurs, it would indicate that methanogens capable of using the competitive substrates are active in these sites. Further, since methane production in these hypersaline environments is limited by substrate concentrations (Kelley et al. 2014), if this limitation was relieved by inhibiting the sulfate reducers, then that condition (the relief of substrate limitation) should be observed in the isotopic values of the methane produced. When more substrate is available to the methanogens, greater isotopic fractionation and so more ¹³C-depleted methane δ¹³C values should result.

Methods and Materials

Three hypersaline field locales were investigated for this work. Two were managed salt ponds, the Don Edwards San Francisco Bay National Wildlife Refuge in Fremont, CA, and the Exportadora de Sal, S.A. de C.V. salterns located in Guerrero Negro, Baja California Sur, Mexico. The third field area was located within the Atacama Desert, Chile.

Ponds 15 and 23 at the Don Edwards site were sampled in January and August 2010. The bottom of Pond 15, with a salinity of ~120 ppt (parts per thousand), consisted of a thin microbial mat overlying ~10 cm of black sediment. At a much higher salinity (~300 ppt), the bottom of Pond 23 was covered with a gypsum/halite crust containing an endoevaporite microbial community. Cores from Pond 15 and pieces of the gypsum/halite crust from Pond 23 were brought back to NASA Ames Research Center where incubations were set up on the day of collection. At the Guerrero Negro salt production area, samples were collected in October 2009, March and November 2012 and March 2014 from Areas 1, 4 and 9, with salinities of about 60 (Area 1), 80 (Area 4) and 170 (Area 9) ppt. The Areas 1 and 4 samples consisted of soft, thick microbial mats, while Area 9 contained endoevaporite gypsum crusts. Incubations of samples from these sites were set up on the day of sampling in Guerrero Negro. The other two sampling sites were located within the Atacama Desert in Chile. The Atacama sites were sampled in May 2012 and May 2013. In May 2012, samples of gypsum crusts were obtained from the largest of many hypersaline pools at Salar de Llamara. The salinity of the overlying water was approximately 130 ppt. In May 2013, the same site at Salar de Llamara was again sampled, as was another gypsum crust at Laguna Cejar (salinity ~77 ppt) within the Salar de Atacama. The gypsum samples were bagged and transported to NASA Ames Research Center. Incubations were initiated within 2 weeks of sampling. At all sites, bubbles were collected. These bubbles ranged in concentrations from 4 to 63% methane (Tazaz et al. 2013) at the Don Edwards and Guerrero Negro sites, whereas bubbles obtained from the Chile sites were all <0.3% methane

(unpublished data). See Kelley et al. (2012, 2014) for maps of the study areas and further descriptions of the sites.

Methane production was determined from incubations of slurries within 38-ml serum vials, using approximately 10–20 g of material (homogenized soft mat or crushed gypsum crust) and 10 ml of deoxygenated (N_2 -purged) site water. In addition, once the vials were sealed, the headspace was also purged with N_2 . For the Pond 15 samples, the thin microbial mat was homogenized with the underlying black sediment. Both control (in which no molybdate was added) and molybdate-amended vials were set up in triplicate for each site. Sodium molybdate was added to inhibit bacterial sulfate reducers, which compete for H_2 and acetate with hydrogenotrophic and acetotrophic methanogens (Banat et al. 1981). Because molybdate acts as a competitive inhibitor for sulfate reduction (Oremland and Capone 1988), sodium molybdate was added at what was calculated to be roughly equivalent concentrations to the ambient sulfate concentration. Site salinity was used to approximate sulfate concentrations in the site water, since sulfate concentrations were not in hand when incubations were set up, assuming that the water at the sites derived from concentrated seawater and based on the sulfate concentration of seawater (29 mM sulfate at 35 ppt). For example, the Cejar3 sample had a site water salinity of 77 ppt, 2.2 times more saline than seawater. Given that salinity, a 64 mM molybdate solution (2.2 times sulfate seawater concentration) was made by dissolving 1.5 g of sodium molybdate dihydrate into 100 ml of site water. This molybdate-amended site water was then used for all the molybdate-amended incubations at that site. Similar calculations were done for the other sites. Although some of the molybdate concentrations were quite high (Table 1), there was no evidence that these concentrations caused problems in the incubations.

Sulfate concentrations for the sites were determined at a later date (Nicholson 2013; Beaudoin 2015). Those sites that are known to be from evaporated seawater (Don Edwards and Guerrero Negro sites) had sulfate concentrations that were within approximately 10% of the molybdate concentrations that were added. The brines at the Atacama Desert sites are derived from evaporated freshwater that has interacted with volcanic rock (Bull and Asenjo 2013). At these sites, the molybdate concentration that was added was about half the measured sulfate concentrations.

All vials were incubated in the dark at room temperature (20–25 °C) for 2–62 days, depending on the rate of methane production. Methane concentrations were measured three to six times during the incubations, with the frequency of headspace sampling adjusted based on how quickly methane concentrations increased in the headspace. The majority of all incubations had four or more time points used in the determination of the production rate. Before headspace samples were analyzed, the vials were shaken to equilibrate the headspace with the methane in the slurry, after which 100 μ L was removed for the measurement. The amount removed for measurement was small compared to the volume of headspace (approximately 10–18 ml). Concentrations were measured with a gas chromatograph equipped with a flame ionization detector and Porapak Q column. The methane production rate was determined from a linear regression of the buildup of methane in the headspace of the incubation vials through time. Methane production rates were normalized to the mass (wet weight) of sample incubated and reported in $nmol\ g^{-1}\ day^{-1}$. Once the methane concentration exceeded ~1000 ppm in the headspace, the incubation was terminated by freezing at –20 °C. The vials were then shipped on ice to either Florida State University or the University of Missouri for isotopic analyses of the headspace methane. At both institutions, the methane $\delta^{13}C$ values were determined using a gas chromatograph with a 60 m PLOT-Q capillary column in line with a ThermoQuest Finnigan IRMS. If the methane concentration was too low for direct injection onto the head of the GC column, a

Table 1 Methane production rates ($\pm$ s.d.) and stable carbon isotope values ($\pm$ s.d.) with and without added sodium molybdate

Site	Date	Salinity (ppt)	Approx molybdate conc (mM)	Methane production (nmol g ⁻¹ day ⁻¹)		Methane δ ¹³ C value (‰)	
				Control	Molybdate	Control	Molybdate
Soft microbial mats							
Pond 15	January 2010	126	104	0.7 ± 0.0	16.1 ± 1.8	-62.8 ± 0.2	-39.9 ± 0.5
Pond 15	August 2010	115	95	0.1 ± 0.0	2.8 ± 0.2	-64.3 ^a	-43.4 ± 0.8
Area 1	October 2009	55	45	15.5 ± 5.7	295.2 ± 27.5	-48.1 ± 3.4	-35.0 ± 0.1
Area 1	March 2012	62	51	5.4 ± 0.7	120.4 ± 62.7	-56.5 ± 1.7	-37.8 ± 13.3
Area 1	November 2012	60	50	22.5 ± 3.8	823.3 ± 45.2	-46.7 ± 1.7	-35.4 ± 2.2
Area 1	March 2014	64	53	86.8 ± 31.8	389.6 ± 68.4	-30.1 ± 1.0	-35.2 ± 1.0
Area 4	October 2009	84	70	2.2 ± 1.4	13.2 ± 4.4	-47.6 ± 2.0	-37.0 ± 1.6
Gypsum-hosted endoevaporite mats							
Pond 23	January 2010	320	265	0.4 ± 0.1	0.5 ± 0.1	-41.5 ± 0.7	-42.3 ± 0.2
Pond 23	August 2010	275	228	2.3 ± 0.2	2.8 ± 0.2	-44.5 ± 1.1	-45.7 ± 1.2
Area 9	October 2009	192	160	19.6 ± 0.8	17.2 ± 1.7	-36.2 ± 2.8	-54.4 ± 2.2
Area 9	March 2014	150	124	0.2 ± 0.1	0.1 ± 0.1	-39.5 ± 13.6	n.d.
Llamaraz2	May 2012	138	114	1.3 ± 0.4	1.6 ± 0.3	-31.1 ± 0.6	-29.5 ± 0.5
Llamaraz3	May 2013	132	109	4.0 ± 2.5	2.4 ± 1.0	-43.9 ± 13.1	-54.7 ± 3.6
Cejar3	May 2013	77	64	1.0 ± 0.1	3.1 ± 0.5	-35.0 ± 4.0	-54.8 ± 2.5

n.d. not determined

^aOnly one vial was measured for $\delta^{13}\text{C}$ of the produced methane

cryo-trapping and focusing method similar to Rice et al. (2001) was used. Working methane isotope standards were inter-calibrated between the laboratories, with the Florida State University standard also calibrated to NBS-19 (a NIST $\delta^{13}\text{C}$ isotope standard).

In addition to the controls and molybdate-amended incubations described above, additional experiments were conducted in March 2012 at Area 1 of the Guerrero Negro site and May 2013 at the Atacama Desert gypsum sites, Llamara3 and Cejar3. These experiments were designed to determine whether competitive substrates were used by methanogens when sulfate reduction was inhibited with molybdate. Control and molybdate-amended vials (as above) were used, as well as additional vials containing molybdate and either 10 μM 99 atom% ^{13}C -labeled bicarbonate or 10 μM 99 atom% ^{13}C -labeled sodium acetate (final concentrations). Bicarbonate concentrations at these sites range from 1 to 4 mM (Des Marais et al. 1989; Kelley et al. 2015), and so a 10 μM addition of labeled bicarbonate should not affect the production. Although the concentrations of acetate were not measured, it was assumed that acetate was also added in trace quantities and so would not affect production rates. Even if this assumption was not strictly true for acetate, which can have low in situ concentrations in marine and hypersaline environments (e.g., King 1991; Kelley et al. 2006), the experiments were designed to determine only whether labeled substrate was taken up by methanogens when sulfate reduction was inhibited. The vials were set up in triplicate, and methane production rates and methane $\delta^{13}\text{C}$ values were determined. Uptake by methanogens of the ^{13}C -labeled substrate would result in the methane $\delta^{13}\text{C}$ value measured from any particular vial to become enriched in ^{13}C , leading to higher values than methane measured from the molybdate-only vials, which contained no label.

Results

Methane production rates from unamended control incubations varied from 0.1 to 86.8 $\text{nmol g}^{-1} \text{ day}^{-1}$ (Table 1) across sample sites, with no apparent relationship between production rates and salinity (Fig. 1). There was also no significant difference in rates of methane produced between the soft microbial mat and gypsum-hosted endoevaporites at the sites (Students *t* test, $p=0.26$).

While there was no significant difference in methane production rates at the sites based on mat type, there was a difference in the stable isotopic values of the methane produced. Across all sites and mat types, methane $\delta^{13}\text{C}$ values ranged from -30.1 to -64.3‰ (Table 1). The soft microbial mat samples averaged $-50.9 \pm 11.7\text{‰}$, and the gypsum crust samples averaged $-38.8 \pm 4.9\text{‰}$. This approximately 10‰ difference in methane $\delta^{13}\text{C}$ values is significantly different (Students *t* test, $p=0.03$).

When molybdate was added to the incubation vials, methane production rates increased significantly (Students *t* test, $p=0.04$) within all the soft mat samples (Table 1), ranging from fourfold to 37-fold greater production than in unamended controls (Fig. 1). In contrast, production rates changed little, if at all, in molybdate-amended gypsum crust samples (Table 1, Fig. 1) with no significant difference between the controls and the molybdate-amended rates (Students *t* test, $p=0.39$).

Along with the large increase in production within the molybdate-amended soft mat samples, the $\delta^{13}\text{C}$ values of the produced methane also increased significantly (Students *t* test, $p=0.01$) (Fig. 1). The average of the molybdate-amended samples for soft mats was $-37.7 \pm 3.1\text{‰}$, over 10‰ higher, more ^{13}C -enriched, than the controls ($-50.9 \pm 11.7\text{‰}$). On the other hand, the $\delta^{13}\text{C}$ values of methane produced by the gypsum crusts samples

Fig. 1 Methane production rates (a, c, e) and methane $\delta^{13}\text{C}$ values (b, d, f) with (open symbols) and without (closed symbols) added molybdate for the Don Edwards (a, b), Guerrero Negro (c, d) and Atacama Desert (e, f) sites. Open and closed circles are soft microbial mats, while open and closed squares are gypsum-hosted endoevaporite mats. Error bars represent $\pm$ s.d

either did not change or became lower than the controls after molybdate amendments, with the molybdate-amended samples averaging $-46.9 \pm 10.0\text{‰}$. As noted above, the controls averaged $-38.8 \pm 4.9\text{‰}$. Overall, this change in $\delta^{13}\text{C}$ values for the gypsum samples was not significant (Student's *t* test, $p=0.09$).

For the substrate ^{13}C -labeling experiment at Area 1 of Guerrero Negro, the production of methane in all of the vials containing molybdate (molybdate only, molybdate plus acetate, molybdate plus bicarbonate) was significantly higher (Student's *t* test, $p=0.0003$) than the control vials (Table 2, Fig. 2). Production in the vials containing molybdate averaged $168 \pm 80 \text{ nmol g}^{-1} \text{ day}^{-1}$ ($n=9$). Comparing production rates in the molybdate-only vials and either molybdate plus ^{13}C -labeled acetate or molybdate plus ^{13}C -labeled bicarbonate shows no significant difference at the $p=0.05$ level. Although it appears that the vials containing molybdate plus ^{13}C -labeled bicarbonate had production rates approximately double of the vials containing molybdate only (Table 2), the large standard deviations on both the values from the molybdate-only vials and those containing both molybdate and ^{13}C -labeled bicarbonate preclude drawing any significance to this.

The methane within the molybdate-amended vials at Area 1 was also enriched in ^{13}C relative to the control vials (Table 2, Fig. 2). To determine whether either acetate or H_2

Table 2 Methane production ($\pm$ s.d.) and methane $\delta^{13}\text{C}$ values ($\pm$ s.d.) obtained from the ^{13}C -labeled substrate experiments

Treatment	Area 1 (soft mat)		Llamara3 (gypsum)		Cejar3 (gypsum)	
	Production ($\text{nmol g}^{-1} \text{day}^{-1}$)	$\delta^{13}\text{C}$ (‰)	Prod ($\text{nmol g}^{-1} \text{day}^{-1}$)	$\delta^{13}\text{C}$ (‰)	Prod ($\text{nmol g}^{-1} \text{day}^{-1}$)	$\delta^{13}\text{C}$ (‰)
Control	5.4 $\pm$ 0.7	-56.5 $\pm$ 1.7	4.0 $\pm$ 2.5	-43.9 $\pm$ 13.1	1.0 $\pm$ 0.1	-35.0 $\pm$ 4.0
Molybdate-only	120.4 $\pm$ 62.7	-37.8 $\pm$ 10.5	2.4 $\pm$ 1.0	-54.7 $\pm$ 3.6	3.1 $\pm$ 0.5	-54.8 $\pm$ 2.5
Moly + ^{13}C -acetate	128.7 $\pm$ 26.8	-33.1 $\pm$ 2.8	4.5 $\pm$ 1.6	-44.3 $\pm$ 11.7	3.2 $\pm$ 0.2	-55.2 $\pm$ 1.8
Moly + ^{13}C -bicarbonate	253.8 $\pm$ 66.1	-21.3 $\pm$ 1.4	2.0 $\pm$ 0.6	-57.2 $\pm$ 2.3	3.3 $\pm$ 0.8	-53.6 $\pm$ 7.4

Fig. 2 Data from the ^{13}C -labeled substrate experiment at the soft mat site of Area 1 in Guerrero Negro. The upper panel shows the methane production rates, while the lower panel shows the methane $\delta^{13}\text{C}$ values for the unamended controls, molybdate-only amendment, molybdate plus $10\ \mu\text{M}$ ^{13}C -labeled sodium acetate amendment and molybdate plus $10\ \mu\text{M}$ ^{13}C -labeled bicarbonate amendment. Error bars represent $\pm$ s.d

(used to reduce CO_2 to CH_4) was being used by the methanogens when sulfate reduction was inhibited, the vials containing the ^{13}C label and molybdate were compared to vials containing only molybdate. The methanogens within the vials containing the ^{13}C -labeled sodium acetate with molybdate did not produce methane that was isotopically different from the methane produced within the molybdate-only vials (Student's t test, $p=0.25$), indicating acetate was not used by methanogens at this site. However, when ^{13}C -labeled sodium bicarbonate was combined with molybdate, the methane produced was enriched in ^{13}C over the molybdate-only vials (Table 2, Fig. 2). Although this enrichment was not statistically significant at the 0.05 level (Student's t test, $p=0.06$), it is significant at the 0.10 level and does suggest some use of the ^{13}C -labeled bicarbonate (and thus H_2).

A different pattern was observed for the ^{13}C -labeling experiments at the Atacama Desert gypsum sites (Table 2). For the Llamara3 site, there was no difference in methane production between the controls and the nine molybdate-amended vials (Student's t test, $p=0.19$). The average methane production overall was $3.2 \pm 1.8\ \text{nmol g}^{-1}\ \text{day}^{-1}$. Similarly, the methane $\delta^{13}\text{C}$ values were not different between the controls and the molybdate-amended vials (Student's t test, $p=0.23$) at Llamara3. The average $\delta^{13}\text{C}$ value overall was $-50.0 \pm 9.9\text{‰}$. Since the isotopic composition for those vials receiving ^{13}C -labeled substrates was not different from the molybdate-only vials (or the controls), it can be concluded that the methanogens at this site did not use the ^{13}C -labeled substrates to produce methane. For the Cejar3 site, methane production in the control vials averaged $1.0 \pm 0.1\ \text{nmol g}^{-1}\ \text{day}^{-1}$ and was significantly lower than the nine molybdate-amended vials, which averaged $3.2 \pm 0.5\ \text{nmol g}^{-1}\ \text{day}^{-1}$ (Student's t test, $p < 0.0001$). Similarly, the methane $\delta^{13}\text{C}$ values of the controls, $-35.0 \pm 4.0\text{‰}$, were also different from the molybdate-amended vials, which averaged $-54.5 \pm 4.1\text{‰}$ (Student's t test, $p < 0.0001$). However, importantly for this experiment, there was no difference in methane $\delta^{13}\text{C}$ values between the molybdate-only vials and the molybdate vials that also contained ^{13}C -labeled bicarbonate (Student's t test, $p=0.80$) or molybdate vials containing ^{13}C -labeled acetate (Student's t test, $p=0.82$),

indicating that neither substrate was taken up by methanogens at Cejar3. Overall at these gypsum sites, neither competitive substrate (H_2/CO_2 or acetate) was used to produce methane, even in the absence of any competition with sulfate reducers.

Discussion

Soft mats and gypsum-hosted endoevaporite mats differed in their responses to molybdate additions, both in methane production rates and in the $\delta^{13}\text{C}$ values of the produced methane (Figs. 1, 3), and so will be discussed separately.

Soft Microbial Mats

Within the soft mats, both methane production and $\delta^{13}\text{C}$ values increased significantly with the addition of molybdate to inhibit sulfate reducers. The large increase in methane production rates was expected based on the previous work at Area 4 of the Guerrero Negro site, in which sulfate reduction was found to be the main anaerobic carbon remineralization process in the mats (Canfield and Des Marais 1993). Methane production occurs at these sites at low rates (Fig. 1) and so, perhaps predictably, when sulfate reduction was inhibited, methane production increased many-fold (Table 1, Fig. 1). With molybdate addition, the methanogens presumably used a competitive substrate, most likely H_2/CO_2 (see below).

The increase in methane $\delta^{13}\text{C}$ values after molybdate inhibition of sulfate reduction, however, was not expected. The previous work at these hypersaline environments has found ^{13}C -enriched methane being produced (Kelley et al. 2012; Tazaz et al. 2013), with the $\delta^{13}\text{C}$ values ranging from about -33 to -72‰ . The higher, more ^{13}C -enriched values came from the higher salinity gypsum sites. These researchers hypothesized that this enrichment was due to substrate limitation. In support of this hypothesis, Kelley et al. (2014) demonstrated that when excess substrate, in the form of the non-competitive trimethylamine, was

Fig. 3 Increase in methane $\delta^{13}\text{C}$ values (control minus molybdate-amended) vs the relative increase in methane production rates between the controls (no amendments) and molybdate-amended vials for the soft microbial mats. The increases in production rates were determined by dividing production rates of molybdate-amended samples by the controls (i.e., a value on the graph of 20 represents a production rate in the molybdate-amended vial 20 times greater than the control vial). The linear regression of the data is also shown. Error bars represent $\pm$ s.d.

added to incubation vials, the methane $\delta^{13}\text{C}$ values fell to lower values by 10 to 71‰ over the controls at all hypersaline sites investigated (both soft mat and endoevaporites). The expectation for the molybdate-amended incubations, therefore, was that the methane $\delta^{13}\text{C}$ values would decrease for these substrate-limited methanogens when additional substrate was made available due to the inhibition of bacterial sulfate reduction. As noted above, this was not the case. Instead, the methane $\delta^{13}\text{C}$ values increased within the molybdate-amended vials.

To understand the observed increase in methane $\delta^{13}\text{C}$ values in the molybdate-amended vials, the relative increase in methane production rates at the soft mat sites was plotted against the increase in methane $\delta^{13}\text{C}$ values (Fig. 3). The linear relationship observed between the increase in methane $\delta^{13}\text{C}$ values and the increase in production rates is statistically significant at the $\alpha=0.05$ level. This correlation suggests that with the increase in substrate concentration freed for use by the methanogens, the response of the methanogen population was swift. Methane production increased rapidly presumably due to an increase in the population and quickly led to even greater substrate limitation. Therefore, with even greater substrate limitation in the molybdate-amended vials, there were less isotopic fractionation and thus the production of more ^{13}C -enriched methane relative to the controls. Future work to determine the species present as well as the increase in the number of *mcrA* gene copies (using qPCR, e.g., García-Maldonado et al. 2015) could be used to test this hypothesis. The *mcrA* gene codes for the enzyme, methyl coenzyme M reductase, that catalyzes the final step in biological methanogenesis; a greater number of gene copies would indicate a growth in the number of methanogens.

Gypsum-Hosted Endoevaporite Mats

There was no significant rise in methane production rates in molybdate-amended gypsum-hosted endoevaporite mats, relative to unamended controls (Table 1, Fig. 1). Along with no discernible change in production with added molybdate, the methane $\delta^{13}\text{C}$ values either stayed the same or became lower than the controls. As noted in the methods, the amount of molybdate added to the Atacama Desert sites was about half the actual sulfate concentrations at these sites. Even so, these gypsum sites responded similarly to the Area 9 gypsum site of Guerrero Negro, where the molybdate was approximately equimolar to the sulfate, suggesting that the molybdate concentrations at the Atacama Desert gypsum sites were sufficient to inhibit the bacterial sulfate reducers.

In the work conducted on gypsum endoevaporite crusts from solar salterns in Israel, Sørensen et al. (2004) found that molybdate successfully inhibited sulfate reduction and stimulated methane production below a salinity of about 180 ppt. Above this salinity, there was no difference in methane production rates measured with and without molybdate additions. Sørensen et al. (2004) also found that methanogens showed a much broader salinity tolerance than sulfate reducers, with little or no sulfate reduction occurring above about 215 ppt (but methane production above that salinity). Based on their results, sulfate reducers would not be active in the Don Edwards Pond 23 samples as the salinity of that site was near 300 ppt. This likely explains our observation that molybdate addition did not change the methane production rate nor methane $\delta^{13}\text{C}$ values at this site.

At both Area 9 of Guerrero Negro and the sites in the Atacama Desert, the methane $\delta^{13}\text{C}$ values generally became more depleted in ^{13}C in molybdate-amended samples relative to the unamended controls. Although the rate of production did not vary much between the molybdate-amended and control vials at these sites, the change in $\delta^{13}\text{C}$ values suggests

that changes in competition between the sulfate reducers and the methanogens did occur. Lower $\delta^{13}\text{C}$ values in the molybdate-amended vials indicate greater isotopic fractionation between the substrate(s) used and the methane produced. As noted above, methanogens in hypersaline environments are thought to be operating under the conditions of substrate limitation (Kelley et al. 2012). For greater fractionation to occur, as observed in the molybdate-amended vials, either the abundance of substrate must be greater so that the fractionation inherent in the enzymatic reactions associated with methane production can be more fully expressed, or there was a change in the mixture of substrate available to the methanogens, since it has been shown that the fractionation between individual substrate and methane produced varies (e.g., Conrad 2005). For example, if the relative concentration of trimethylamine, which has a large methane fractionation factor (Summons et al. 1998), increased with no change in the absolute substrate concentration, the methane $\delta^{13}\text{C}$ value may become depleted without the overall production rate changing.

Methane Production from ^{13}C -Labeled Competitive Substrates

These experiments were undertaken to determine which, if any, competitive substrate was used by methanogens when sulfate-reducing bacteria were inhibited by molybdate. It was geared mainly to the Guerrero Negro Area 1 site, a soft mat site, since it was noticed that in October 2009 methane production increased dramatically when molybdate was added to samples of this site. However, two gypsum sites, Llamara3 and Cajar3 of the Atacama Desert in Chile, were also tested for completeness. At neither of the gypsum sites was uptake of ^{13}C -labeled substrates observed (Table 2). If the labeled substrates had been used to any great extent, the $\delta^{13}\text{C}$ values of the vials receiving them would have been higher (more ^{13}C -enriched) than the vials receiving molybdate alone. Apparently, neither H_2 nor acetate is used by methanogens at these sites, even if they become available. This suggests that methanogens capable of using the competitive substrates are not present at these high-salinity gypsum sites.

At Area 1 of Guerrero Negro, there was no difference in methane $\delta^{13}\text{C}$ values between the vials receiving only molybdate and the molybdate-amended vials that also received ^{13}C -labeled acetate. From these results, again it appears that acetate, even if available, is not used by the methanogens. In contrast, when ^{13}C -labeled bicarbonate was added to the molybdate-amended vials, the methane $\delta^{13}\text{C}$ value did increase above the molybdate-only vials indicating bicarbonate (i.e., H_2/CO_2) use. Of the competitive substrates, acetate and H_2 , evidently only the H_2 is used by the methanogens in these hypersaline environments.

Although methane production via the use of H_2/CO_2 by methanogens does apparently increase at the Area 1 site, H_2/CO_2 alone cannot account for the large increase in methane production that was observed when sulfate reducers were inhibited by molybdate. If it had, the $\delta^{13}\text{C}$ values of the methane produced should have been much higher (on the order of +300‰). From the measured $\delta^{13}\text{C}$ values in the molybdate plus ^{13}C -labeled bicarbonate vials, only approximately 4% of the total methane produced came from H_2/CO_2 based on isotopic mass balance considerations similar to those used in Kelley et al. (2015).

What else might be contributing to the large increase in methane production when molybdate is added? We hypothesize that the increase may be related to glycine betaine, one of the compounds that sulfate reducers use for osmotic regulation in hypersaline environments (Welsh et al. 1996). Glycine betaine is a precursor to trimethylamine, which has been shown to be the main substrate used by methanogens at all of these sites, contributing 60–90% of methane produced (Kelley et al. 2015). When the sulfate reducers are inhibited

by molybdate, they are not producing or taking up glycine betaine from the environment. This then leaves more glycine betaine (which again degrades to trimethylamine) for the methanogens, which are poised to rapidly use it, thus increasing methane production.

Another consideration as to why the competitive substrates were not used to a greater extent when sulfate reducers were inhibited may be the methanogen community structure. The methanogen community, and thus substrate use, can be controlled by salinity because of energy constraints, with acetate used at lower salinities (generally not above approximately 60 ppt) than H_2/CO_2 (Oren 2011). The ^{13}C -labeled substrate data are also corroborated by genetic work. The most prevalent methanogens identified in the salt works in Guerrero Negro are those within the Methanosarcinaceae Family, with the methylotrophic *Methanohalophilus* being the dominant genus (García-Maldonado et al. 2012, 2015). These methanogens use the non-competitive substrates abundant at the sites. In addition, members of the Order Methanomicrobiales and Methanobacteriales, putative hydrogenotrophs, were also found at the Guerrero Negro sites (García-Maldonado et al. 2015, 2018), suggesting methanogens capable of using H_2 are also present at these sites.

Conclusions

This work investigated the competition between sulfate reducers and methanogens across different microbial mat types located in diverse hypersaline environments. This competition was studied by inhibiting bacterial sulfate reduction with molybdate and measuring changes in methane production rates and methane $\delta^{13}C$ values. Different responses to the molybdate amendments were observed. At the highest salinity endoevaporite sites (e.g., Pond 23), molybdate additions resulted in no change in methane production or methane $\delta^{13}C$ values, indicating that sulfate reducers are not active at these very high salinities. At the somewhat lower salinity endoevaporite sites (e.g., Area 9), there was no significant increase in methane production, although there was a decrease in the methane $\delta^{13}C$ values. This suggests that there was some substrate freed up by the inhibition that the methanogens were able to use. This minimal increase in the substrate pool allowed for greater isotopic fractionation and so lower methane $\delta^{13}C$ values. At the lower salinity, soft microbial mat sites, methane production increased dramatically in the molybdate-amended vials indicating that the methanogen community was poised to rapidly exploit substrate when it became available. The methane $\delta^{13}C$ values at these sites also increased probably due to even greater substrate limitation resulting from the high methane production rates.

From our data, it appears that the well-known competition between bacterial sulfate reducers and methanogens is most active in the lower salinity brines and soft microbial mat environments, with the competition being for H_2 . At the higher salinities in the gypsum-hosted endoevaporite mats, little competition, if any, occurs, either because acetotrophic or hydrogenotrophic methanogens are not present, or because the sulfate reducers are not active. The high ^{13}C -enriched methane $\delta^{13}C$ values also indicate that if methane isotope values alone are used to determine the biogenicity of methane on Mars (or other planets/moons), false negatives may result as biogenic methane can be produced with atypically enriched ^{13}C values. We would recommend the use of methane δ^2H values, along with $\delta^{13}C$ values, since the hydrogen stable isotopes do not seem to be affected to the same degree (e.g., Tazaz et al. 2013).

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